In-vitro anti-tumor activity studies of bridged and unbridged benzyl-substituted titanocenes

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The benzyl-substituted *ansa*-titanocenes [1,2-di(cyclopentadienyl)-1,2-di-(4-*N*,*N*-dimethylaminophenyl)ethanediyl] titanium dichloride (Titanocene X) and [1,2-di(cyclopentadienyl)-1,2-bis(*m*-dimethoxyphenyl)ethanediyl] titanium dichloride (Titanocene Z), and the benzyl-substituted unbridged titanocene bis-[(*p*-methoxybenzyl)cyclopentadienyl] titanium(IV) dichloride (Titanocene Y) were tested on the growth of a wide variety of tumor cells *in vitro* on a panel of 36 human tumor cell lines containing 14 different tumor types investigated in a cellular proliferation assay. Titanocene Y with a mean IC_{50} value of 65.8×10^{-6} mol/l over the full panel of 36 cancer cell lines reaches the activity of cisplatin with 14.7×10^{-6} mol/l within a factor of 4, whereas Titanocene X and Z show significantly less cytotoxic activity. Titanocene Y is most effective on pleura mesothelioma, and uterine and renal cell cancer, where the IC_{50} values are comparable or significantly better than for cisplatin. In particular, in the case of renal cell cancer and pleura mesothelioma there is an obvious lack of chemotherapeutic reagents, which might be filled by Titanocene Y, where a very promising cytotoxic effect in comparison with cisplatin could be shown. *Anti-Cancer Drugs* **16:1091–1098 © 2005 Lippincott Williams & Wilkins.**

Anti-Cancer Drugs 2005, **16**:1091–1098

Keywords: anti-cancer drug, cisplatin, human tumor cell lines, renal cell cancer, titanocene

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Sponsorship: The authors thank Science Foundation Ireland for funding through grant (04/BRG/C0682). In addition, funding from the Higher Education Authority (HEA) and the Center for Synthesis and Chemical Biology through the HEA PRTLI cycle 3 as well as COST D20 (WG 0001) was provided.

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Received 14 June 2005 **Accepted** 4 August 2005

Introduction

Despite the resounding success of cisplatin and closely related platinum anti-tumor agents, the movement of other transition metal anti-cancer drugs towards the clinic has been exceptionally slow [1–3]. Metallocene dichlorides (Cp_2MCl_2) with M = Ti, V, Nb and Mo show remarkable anti-tumor activity [4,5]. Unfortunately, the efficacy of Cp_2TiCl_2 in phase II clinical trials in patients with metastatic renal cell carcinoma [6] or metastatic breast cancer [7] was too low to be pursued. Very recently, more synthetic effort has been employed to increase the cytotoxicity of titanocene dichloride derivatives [8–12]. A novel method starting from titanium dichloride and fulvenes [13–16] allows direct access to highly substituted *ansa*-titanocenes [17–20] – titanocenes containing a carbon–carbon bridge. By using this method we have synthesized [1,2-di(cyclopentadienyl)-1,2-di-(4-*N*,*N*-dimethylaminophenyl)ethanediyl] titanium dichloride (Titanocene **X**), which has an IC_{50} value of 2.7×10^{-4} mol/l when tested for cytotoxic effects on the LLC-PK cell line [21]. It was followed by reports about heteroaryl [22] and methoxyphenyl [23,24] substituted *ansa*-titanocenes, which show similar IC_{50} values. Our most cytotoxic *ansa*-titanocene [1,2-di(cyclopentadienyl)-1,2-bis(*m*-dimethoxyphenyl)ethanediyl] titanium dichloride (Titanocene **Z**) shows an IC_{50} value of 2.1×10^{-4} mol/l when tested on the LLC-PK cell line [23].

The cytotoxic effect could be increased by synthesizing the analogous unbridged titanocenes by establishing a completely new synthetic route, which has been published recently [25]. Bis-[(*p*-methoxybenzyl)cyclopentadienyl] titanium(IV) dichloride (Titanocene **Y**), which has an IC_{50} value of 2.1×10^{-5} mol/l when tested on the LLC-PK cell line, was synthesized using this new method.

In order to study the direct effects of the *ansa*-Titanocenes **X** and **Z** and the unbridged Titanocene **Y** (see Fig. 1) in comparison to cisplatin on the growth of a wide variety of tumor cells *in vitro*, a panel of 36 human tumor cell lines containing 14 different tumor types was investigated in a cellular proliferation assay and the results are presented in this paper.

Materials and methods

Cell lines

Twenty-four cell lines were established from human tumor xenografts as described by Roth *et al.* [26]. The origin of the donor xenografts was described by Fiebig

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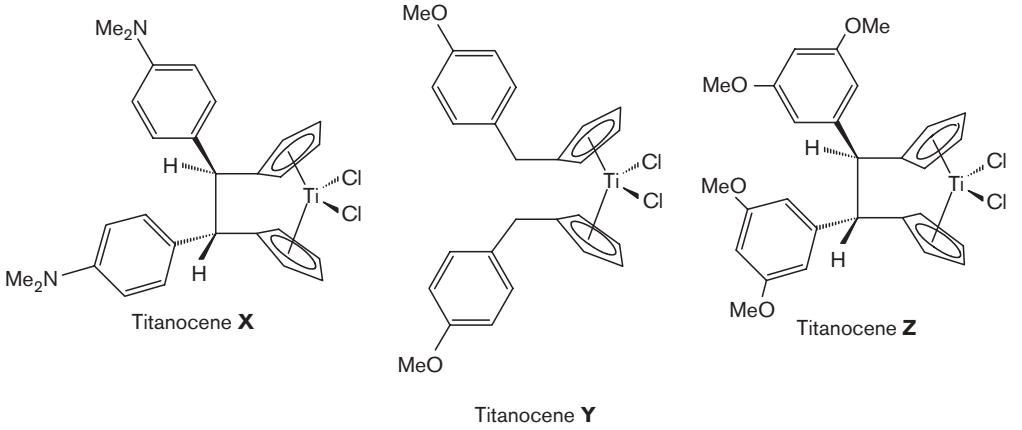
Fig. 1



Molecular structure of Titanocenes **X**, **Y** and **Z**.

et al. [27]. They comprise the following tumor types: BXF 1218L (bladder), CNXF 498NL (CNS), GXF 251L (gastric), HNXF 536L (head and neck), LXF 1121L, LXF 289L, LXF 526L, LXF 629L and LXF 529L (lung, non-small cell), MAXF 401NL (mammary), MEXF 276L, MEXF 394NL, MEXF 462NL, MEXF 520L and MEXF 514L (melanoma), OVXF 1619L and OVXF 899L (ovarian), RXF 1781L, RXF 486L, RXF 944L and RXF 393NL (renal), PAXF 1657L (pancreas), PXF 1752L (pleura mesothelioma), and UXF 1138L (uterus). The cell lines CNXF SF268 (CNS), CXF HCT116 and CXF HT29 (colon), LXF H460 (lung, non-small cell), MAXF MCF7 (mammary), OVXF OVCAR3 (ovarian), and PRXF DU145 and PRXF PC3M (prostate) were kindly provided by the US National Cancer Institute. The cell lines BXF T24 (bladder), PAXF PANC1 (pancreas) and PRXF 22RV1 (prostate) were kindly provided by the ATCC (Manassas, Virginia, USA), whereas the prostate cell line PRXF LNCAP was kindly provided by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany).

Cells were routinely passaged once or twice weekly. They were maintained in culture for no longer than 20 passages. All cells were grown at 37°C in a humidified atmosphere (95% air/5% CO_2) in RPMI 1640 medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS (Sigma, Deisenhofen, Germany) and 0.1% gentamicin (Invitrogen).

Cell proliferation assay

A modified propidium iodide (PI) assay was used to assess the effects of Titanocenes **X**, **Y**, **Z** and cisplatin on the growth of the human tumor cell lines [28].

Briefly, cells were harvested from exponential phase cultures by trypsinization, counted and plated in 96-well flat-bottomed microtiter plates at a cell density dependent on the cell line (4000–10 000 viable cells/well). After 24 h recovery to allow the cells to resume exponential growth, 10 µl culture medium (six control wells per plate) or culture medium containing Titanocenes **X**, **Y**, **Z** or cisplatin were added to the wells. Each concentration was plated in triplicate. Titanocenes and cisplatin preparations were applied in five concentrations ranging from 0.01 to 100 µg/ml. Following 4 days of continuous drug exposure, cell culture medium with or without drug was replaced by 200 µl of an aqueous PI solution (7 µg/ml). Since PI only passes leaky or lysed cell membranes, DNA of dead cells can be stained and measured, while living cells will not be stained. To measure the proportion of living cells, cells were permeabilized by freezing the plates, which resulted in death of all cells. After thawing of the plates, fluorescence was measured using the Cytofluor 4000 microplate reader (excitation 530 nm, emission 620 nm), giving a direct relationship to the total cell number. The assay included an untreated control and the positive reference compound doxorubicin.

Growth inhibition was expressed as treated/control × 100 (%T/C). IC_{50} (Tables 1–3), IC_{70} and IC_{90} values were determined by plotting median T/C values of three independent experiments over compound concentration. Coefficient of variation [SD/mean × 100 (%)] was below 20% in nearly all experiments. Anti-tumor activity was evaluated based on the IC_{50} values (Figs 2–4). The IC_{70} and IC_{90} values were also acquired and can be found in Tables 4 and 5.

Titanocene and cisplatin preparations

Titanocenes **X** and **Z** were synthesized according to [21,23] by reacting the corresponding fulvenes with titanium dichloride, while Titanocene **Y** was prepared from titanium tetrachloride and 2 mol/l of a substituted

Table 1 IC_{50} values ($\times 10^6$ mol/l) of Titanocenes X, Y, Z and cisplatin for BXF, CNXF, CXF, GXF, HNXF, LXF and MEXF

	Titanocene **X**	Titanocene **Y**	Titanocene **Z**	Cisplatin
BXF 1218L	76.6	54.7	80.3	2.79
BXF T24	99.2	56.4	100	6.83
CNXF 498NL	237	78.3	–	8.02
CNXF SF268	56.9	27.3	69.1	2.54
CXF HCT116	90.6	73.2	–	3.79
CXF HT29	537	83.6	–	24.5
GXF 251L	347	84.5	354	8.62
HNXF 536L	71.0	36.9	55.9	4.81
LXF 1121L	65.3	66.6	130	6.79
LXF 289L	108	78.5	247	41.7
LXF 526L	138	87.0	564	5.97
LXF 529L	111	38.7	93.7	10.1
LXF 629L	130	60.6	90.7	9.38
LXF H460	85.4	73.4	168	13.5
MEXF 276L	119	102	152	20.5
MEXF 394NL	110	68.6	215	2.81
MEXF 462NL	597	82.6	326	3.38
MEXF 514L	104	70.0	107	14.3
MEXF 520L	195	43.6	133	15.7

Table 2 IC_{50} values ($\times 10^6$ mol/l) of Titanocenes X, Y, Z and cisplatin for OVXF, PAXF, PRXF and MAXF

	Titanocene **X**	Titanocene **Y**	Titanocene **Z**	Cisplatin
OVXF 1619L	295	80.9	145	4.13
OVXF 899L	380	64.8	–	5.11
OVXF OVCAR3	–	60.9	–	165
PAXF 1657L	–	125	–	34.3
PAXF PANC1	195	90.4	183	29.2
PRXF 22RV1	206	56.1	118	6.82
PRXF DU145	128	29.6	93.9	20.0
PRXF LNCAP	129	50.8	49.8	68.1
PRXF PC3M	120	83.7	75.7	33.3
MAXF 401NL	79.7	75.8	148	1.95
MAXF MCF7	116	76.1	343	46.9

Table 3 IC_{50} values ($\times 10^6$ mol/l) of Titanocenes X, Y, Z and cisplatin for PXF, UXF and RXF

	Titanocene **X**	Titanocene **Y**	Titanocene **Z**	Cisplatin
PXF 1752L	148	77.1	103	185
UXF 1138L	55.8	54.4	75.3	86.0
RXF 1781L	234	47.2	112	192
RXF 393NL	510	120	–	43.9
RXF 486L	–	59.9	–	–
RXF 944L	122	81.1	82.1	27.1
Mean value	149	65.8	141	14.7

lithium cyclopentadienide [25]. Cisplatin was obtained commercially from Sigma (no. P 4394).

Results and discussion

The effects of the three titanocenes in comparison to cisplatin on tumor growth were investigated in a panel of 36 human tumor cell lines comprising 14 different tumor types [2999,30]. Titanocene **X**, **Y** and **Z** and cisplatin were applied in dose levels ranging from 0.01 to 100 μg/ml. The results are summarized in Figs 2–4 for the three titanocenes and cisplatin.

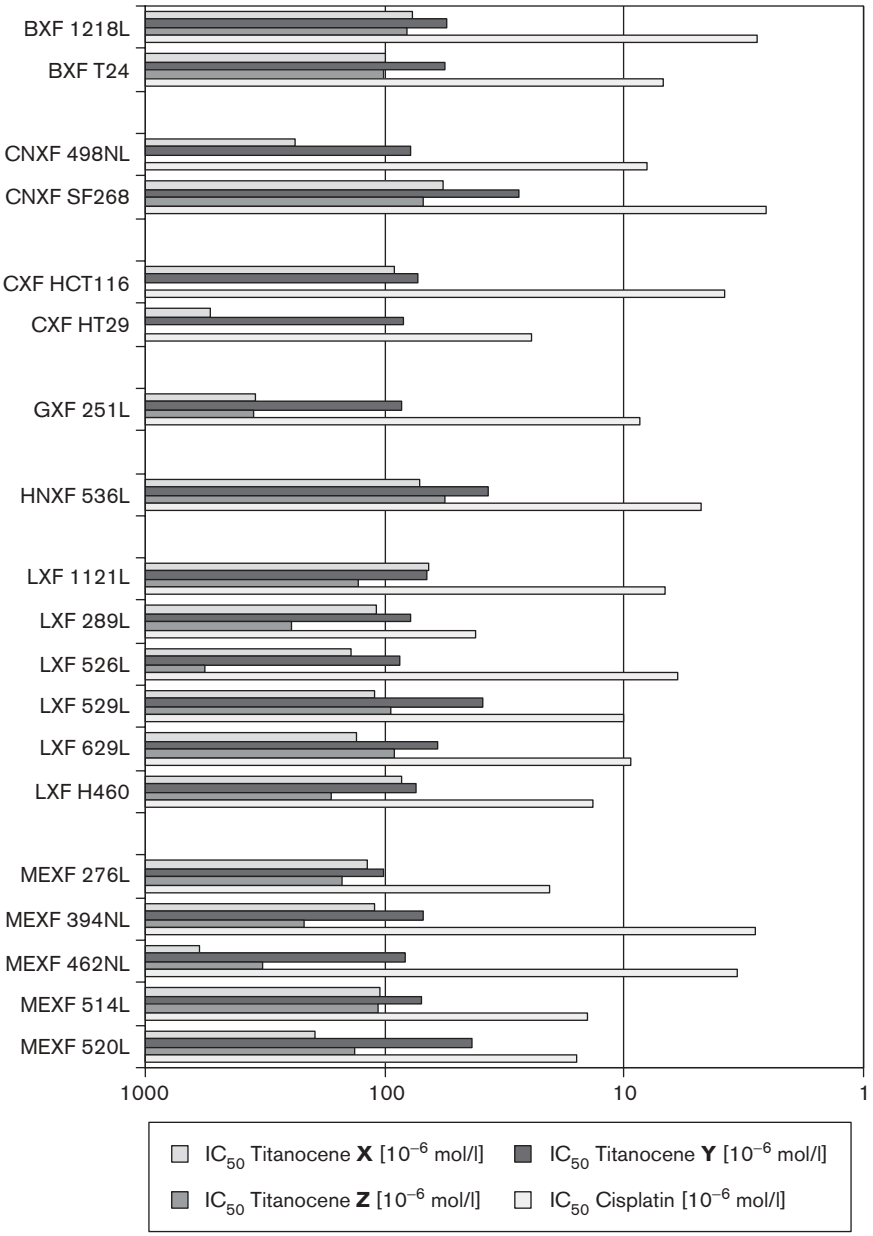
In relation to the cytotoxic effect, the presented results can be divided into three main groups:

The experimental results showing a significantly less cytotoxicity of all three titanocenes in comparison to cisplatin when tested on the tumor types BXF (bladder), CNXF (CNS), CXF (colon), GXF (gastric) HNXF (head and neck), LXF (lung) and MEXF (melanoma) are summarized in the first group (see Fig. 2). These results agree with previously reported ex vivo experiments of Titanocene **X** on freshly explanted tumor cells. The lowest cytotoxic effect compared to the activity on other tumors could be observed when freshly explanted melanoma cancer cells were treated with this titanocene. Concerning this experimental data, there is no obvious application of the titanocenes as possible anti-cancer drugs in this group of tumors.

In the second group, results are presented where an interesting cytotoxic effect on single cell lines can be observed, but where there is no unified effect on the whole tumor type (see Fig. 3). For ovarian cancer cells, cisplatin is mostly more effective when tested on the above-mentioned three cell lines than the titanocenes, except cell line OVXF OVCAR3 where the unbridged Titanocene **Y** with an IC_{50} value of 60.9×10^{-6} mol/l is significantly better than cisplatin (IC_{50} 165×10^{-6} mol/l). A similar pattern can be observed for prostate cancer; cisplatin shows a higher activity compared to the titanocenes when tested on the fast-growing prostate cancer cell line PRXF 22RV1, whereas the cytotoxic effect of the unbridged Titanocene **Y** is as good or even better on the slower-growing cell lines PRXF DU145 and PRXF LNCAP with IC_{50} values of 29.6×10^{-6} and 50.8×10^{-6} mol/l (cisplatin: IC_{50} 20.0×10^{-6} and 68.1×10^{-6} mol/l). For the widespread prostate cancer cell line PRXF PC3M, cisplatin has a higher cytotoxic effect (IC_{50} 33.3×10^{-6} mol/l) than *ansa*-Titanocene **X** (IC_{50} 120×10^{-6} mol/l), and even the effects of Titanocene **Y** and **Z** are lower (IC_{50} 83.7×10^{-6} and 75.1×10^{-6} mol/l). These findings have important implications in determining a mechanism by which these compounds mediate their effects, which may be different from the classical chemotherapeutic agents that induce death in highly proliferative cells where these novel titanocene compounds may mediate their effects independent of growth.

When the pancreas cancer cell line PAXF PANC1 is treated with our titanocenes and cisplatin there is a slight advantage with the unbridged Titanocene **Y** (IC_{50} 90.4×10^{-6} mol/l) within the group of the three titanocenes (Titanocene **X** IC_{50} 195×10^{-6} mol/l,

Fig. 2

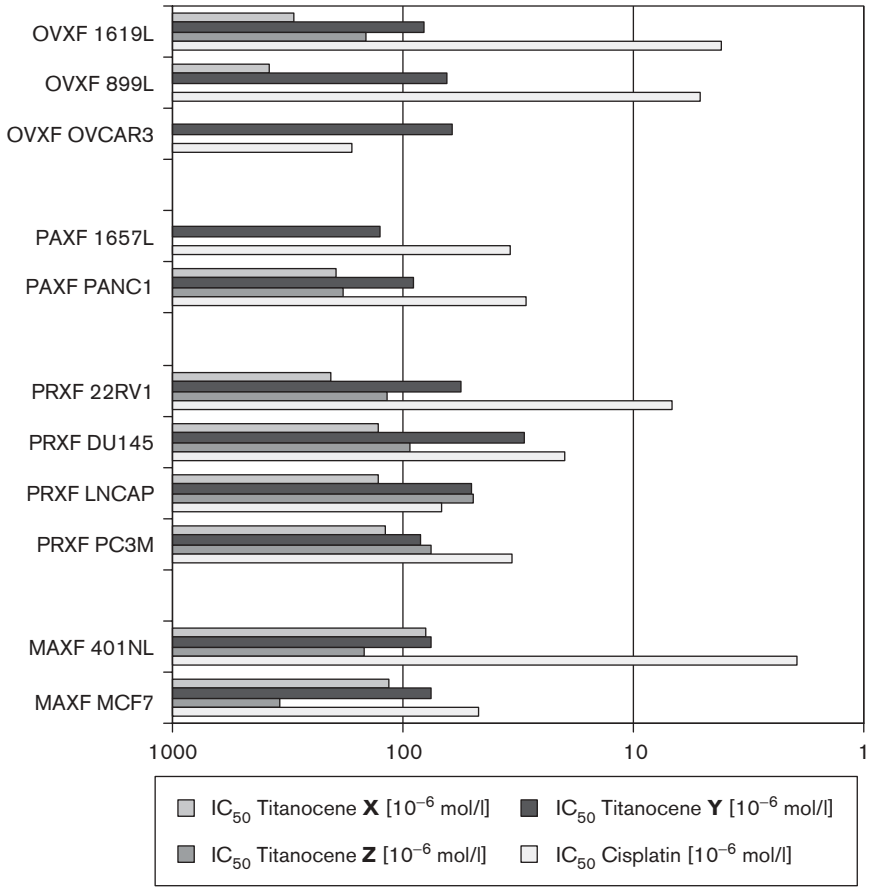


IC_{50} values for Titanocenes **X**, **Y**, **Z** and cisplatin for the tumor types BXF, CNXF, CXF, GXF, HNXF, LXF and MEXF.

Titanocene **Z** IC_{50} 183×10^{-6} mol/l), but all three titanocenes are less effective than cisplatin (IC_{50} 29.2×10^{-6} mol/l). For the second cell line tested (PAXF 1657L), Titanocene **Y** is the only titanocene showing an obvious cytotoxic effect, with an IC_{50} of 125×10^{-6} mol/l, although this is lower than the IC_{50} of cisplatin (IC_{50} 34.3×10^{-6} mol/l). The experimental data agree with previously performed *ex vivo* experiments with freshly explanted pancreas cancer cells, where moderate cytotoxic effects for Titanocene **X** could be observed.

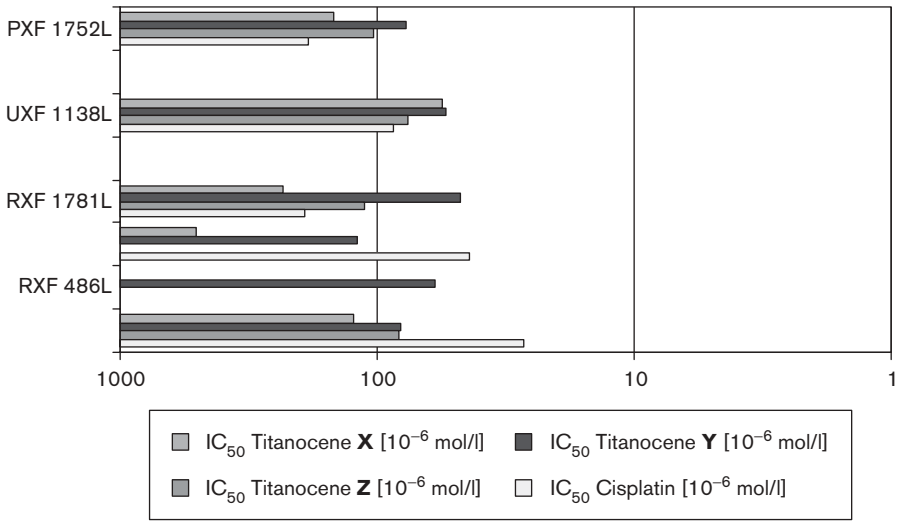
The results from the experiments when the three titanocenes and cisplatin were tested on mammalian cancer cells again show a non-unified pattern. Cisplatin, when tested on the cell line MAXF401NL, is the most effective compound with an IC_{50} value of 2.0×10^{-6} mol/l. Experiments performed on the cell line MAXF MCF7 show a comparable IC_{50} value for cisplatin (IC_{50} 46.9×10^{-6} mol/l) of 76.1×10^{-6} mol/l for Titanocene **Y**, whereas Titanocenes **X** and **Z** are significantly less effective with IC_{50} values of 116×10^{-6} and 343×10^{-6} mol/l, respectively. The good efficacy of our titanocenes

Fig. 3



IC_{50} values for Titanocenes **X**, **Y**, **Z** and cisplatin for the tumor types OVXF, PAXF, PRXF and MAXF.

Fig. 4



IC_{50} values for Titanocenes **X**, **Y**, **Z** and cisplatin for the tumor types PXF, UXF and RXF.

Table 4 IC_{70} values ($\times 10^6$ mol/l) of Titanocenes X, Y, Z and cisplatin

	Titanocene **X**	Titanocene **Y**	Titanocene **Z**	Cisplatin
BXF 1218L	157	116	142	8.15
BXF T24	195	104	196	25.8
CNXF 498NL	–	130	–	34.7
CNXF SF268	109	71.3	125	6.37
CXF HCT116	172	120	–	8.05
CXF HT29	–	136	–	79.8
GXF 251L	–	172	–	89.5
HNXF 536L	143	90.9	115	6.97
LXF 1121L	139	117	279	40.2
LXF 289L	227	149	–	136
LXF 526L	275	157	–	22.6
LXF 529L	284	85.6	217	55.6
LXF 629L	292	119	198	90
LXF H460	154	121	400	56.8
MAXF 401NL	187	134	303	19.2
MAXF MCF7	–	130	–	169
MEXF 276L	271	277	323	66.8
MEXF 394NL	225	122	483	7.82
MEXF 462NL	–	158	–	11.5
MEXF 514L	184	129	194	57.8
MEXF 520L	–	92.7	333	46.9
OVXF 1619L	–	182	471	16.5
OVXF 899L	–	140	–	18
OVXF OVCAR3	–	141	–	>>100
PAXF 1657L	–	267	–	145
PAXF PANC1	500	151	430	95.2
PRXF 22RV1	586	115	314	58.8
PRXF DU145	327	84.2	183	46.9
PRXF LNCAP	244	102	136	254
PRXF PC3M	270	140	142	184
PXF 1752L	444	155	222	>>100
RXF 1781L	–	104	299	>>100
RXF 393NL	–	225	–	117
RXF 486L	–	113	–	>>100
RXF 944L	219	142	138	81.1
UXF 1138L	110	109	145	251
Mean value	227	130	228	46.5

Table 5 IC_{90} values ($\times 10^6$ mol/l) of Titanocenes X, Y, Z and cisplatin

	Titanocene **X**	Titanocene **Y**	Titanocene **Z**	Cisplatin
BXF 1218L	323	247	250	218
BXF T24	384	193	382	142
CNXF 498NL	–	216	–	180
CNXF SF268	208	186	225	68.8
CXF HCT116	326	195	–	67.3
CXF HT29	–	220	–	260
GXF 251L	–	349	–	>>100
HNXF 536L	287	224	236	10.1
LXF 1121L	296	205	–	254
LXF 289L	476	282	–	>>100
LXF 526L	547	283	–	226
LXF 529L	–	189	500	306
LXF 629L	–	235	432	>>100
LXF H460	278	200	–	240
MAXF 401NL	439	236	–	247
MAXF MCF7	–	222	–	>>100
MEXF 276L	–	–	–	218
MEXF 394NL	463	217	–	101
MEXF 462NL	–	302	–	148
MEXF 514L	326	239	354	233
MEXF 520L	–	197	–	140
OVXF 1619L	–	407	–	85.7
OVXF 899L	–	301	–	319
OVXF OVCAR3	–	325	–	>>100
PAXF 1657L	–	568	–	>>100
PAXF PANC1	–	251	–	310
PRXF 22RV1	–	237	–	>>100
PRXF DU145	–	240	357	110
PRXF LNCAP	463	205	369	>>100
PRXF PC3M	605	233	267	>>100
PXF 1752L	–	311	478	>>100
RXF 1781L	–	231	–	>>100
RXF 393NL	–	423	–	312
RXF 486L	–	211	–	>>100
RXF 944L	393	249	231	243
UXF 1138L	216	220	281	>>100
Mean value	361	250	324	164

especially when tested on the widespread mammalian cancer cell line MAXF MCF7, for which even phase II clinical trials of dicyclopentadiene titanium dichloride have been carried out, could point to an interesting potential for further in-vivo experiments in the future.

The third group of results is formed by the experiments performed with the tumor types PXF (pleura mesothelioma), UXF (uterus) and RNXF (renal cell cancer), and combines very promising experimental data concerning the cytotoxic effects of our titanocenes in comparison with cisplatin (see Fig. 4). The IC_{50} values shown in this group are nearly as good as or often better than cisplatin. For uterine cancer, all three titanocenes show a similar cytotoxic effect when tested on the cell line UXF 1138L, especially Titanocenes **X** and **Y** with IC_{50} values of 55.8×10^{-6} and 54.4×10^{-6} mol/l (even Titanocene **Z** IC_{50} 75.3×10^{-6} mol/l) are more cytotoxic than cisplatin (IC_{50} 86.0×10^{-6} mol/l). Very promising results could also be obtained by the treatment of the pleura mesothelioma cancer cell line PXF 1752L with Titanocene **Y**. The experimental data are significantly better than the results achieved by cisplatin (IC_{50} 185×10^{-6} mol/l). The unbridged Titanocene **Y** with an IC_{50} value of 77.1×10^{-6} mol/l is in this case the best titanocene, whereas the *ansa*-Titanocenes **X** and **Z** show the same cytotoxic activity as cisplatin (IC_{50} 148×10^{-6} and 103×10^{-6} mol/l).

Special attention in this experimental series was directed towards renal cell cancer. This tumor type cannot be treated with cisplatin because of nephrotoxic side-effects and phase II studies of clinical trials have already been carried out for dicyclopentadiene titanium dichloride. Therefore, all three titanocenes and cisplatin were tested on four different renal cell cancer cell lines. All three titanocenes show a similar cytotoxic activity (IC_{50} values of 122×10^{-6}, 81.1×10^{-6} and 82.1×10^{-6} mol/l) when tested on the cell line RXF 944L, whereas the cytotoxicity of cisplatin is better (IC_{50} 27.1×10^{-6} mol/l). All three titanocenes show a very low cytotoxicity when tested on the cancer cell line RXF 393NL, in contrast to cisplatin (IC_{50} 43.9×10^{-6} mol/l). The unbridged Titanocene **Y** is again the most effective titanocene with an IC_{50} value of 47.2×10^{-6} mol/l when tested on the renal cell cancer cell line RXF 1781L,

whereas both *ansa*-Titanocenes **X** and **Z** showing IC_{50} values of 234×10^{-6} and 112×10^{-6} mol/l have a very low activity. However, it is important to mention that cisplatin has a very low cytotoxicity with an IC_{50} value of 192×10^{-6} mol/l when tested on this cell line as well and Titanocene **Y** is significantly better. The most promising results were obtained for the renal cell cancer cell line RXF 486L. Again, both *ansa*-Titanocenes **X** and **Z** show very low or nearly no cytotoxic activity when tested on this cell line. Even cisplatin shows nearly no cytotoxicity in contrast to the unbridged Titanocene **Y**, which seems to have a very promising effect on this cancer cell line with an IC_{50} value of 59.9×10^{-6} mol/l. Thus, this cancer cell line and cancerous renal cells, in general, seem to be the most interesting target for an application of the presented titanocenes. These results were underlined by previously published ex-vivo experiments performed with freshly explanted renal cell tumors. In these studies, renal cell cancer was the most interesting target compared with other tumor types for Titanocene **X** as well. The very good efficacy of our titanocenes when tested on renal cell cancer cells, especially in contrast to cisplatin, points to this tumor type as an ideal candidate for further in-vivo experiments in the very close future.

Conclusion and outlook

The evaluation of the Titanocenes **X**, **Y** and **Z** in comparison with cisplatin against 36 cancer cell lines shows the high cytotoxic potential of these three titanocene dichloride derivatives. The mean IC_{50} values summarized over the full panel of the 36 cell lines are 149 and 141×10^{-6} mol/l for Titanocene **X** and **Z**, while Titanocene **Y** with 65.8×10^{-6} mol/l reaches cisplatin with 14.7×10^{-6} mol/l within a factor of 4.

Nevertheless, against bladder, colon, gastric, head and neck, and lung cancer, as well as melanoma, the titanocenes cannot compete against the cytotoxicity of cisplatin. However, in the group of ovarian, pancreas, prostate and breast cancers, individual cell lines were identified which show comparable sensitivity between the best titanocene and cisplatin. The performance of Titanocene **Y** against PRXF LNCAP cells, against which an IC_{50} value of 29.6×10^{-6} mol/l is found, is outstanding. In a final group of pleura mesothelioma, and uterine and renal cell cancer, the cytotoxicity of Titanocene **Y**, in particular, is comparable or better than cisplatin, which underlines the potential for future in-vivo experiments. From these results and the phase II clinical studies performed with titanocene dichloride itself, xenograft mouse models using a renal cell cancer line and the breast carcinoma cell line MCF-7 are our next targets.

In general, one can say that Titanocene **Y** is the best of the three titanocenes and shows a good uniform cytotoxic response against a wide variety of cancer cells. In some cases, it may be able to compete against already established anti-cancer reagents like cisplatin; in the case of renal cell cancer, there is an obvious lack of chemotherapeutic reagents, which might be filled with Titanocene **Y**. Another hope for the future is the establishment of Titanocene **Y** as a second line of defense against platinum (or other)-resistant cancer types, which will become a target for further research.

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